



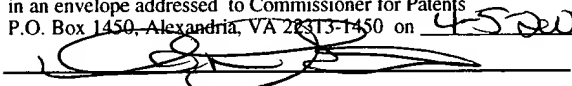
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT : Jackowski et al.  
INVENTION : **CENP-E Biopolymer Marker**  
Indicative of Alzheimer's Disease  
SERIAL NUMBER : 09/993,399  
FILING DATE : November 23, 2001  
EXAMINER : Turner, Sharon L.  
GROUP ART UNIT : 1649  
OUR FILE NO. : 2132.091

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CERTIFICATE UNDER 37 CFR 1.8(a)

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DECLARATION UNDER 37 CFR § 1.132

I, Ferris H. Lander, do hereby declare as follows:

1. I am a registered Patent Agent and am authorized to represent the inventor's and assignee in the application entitled "**CENP-E Biopolymer Marker Indicative of Alzheimer's Disease**", having U.S. Application Serial No. 09/993,399, filed November 23, 2001.

2. In the Office Action mailed on December 20, 2005, claims 1 and 44 (in part) were rejected under 35 USC 101 because the claimed invention allegedly is not supported by either a specific

and substantial asserted utility or a well-established utility.

Claims 1 and 44 (in part) were also rejected under 35 U.S.C. 112, first paragraph. The Examiner asserted that since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility, one skilled in the art would clearly not know how to use the claimed invention.

Specifically, the Examiner asserted that the molecular weight bands of the gel shown in Figure 1 are not visible.

3. The attached figure was produced by scanning the original photograph of the gel. The figure is entitled "DEAE 5(E) Ad vs. Age Matched AD" and represents Figure 1 as originally filed. No new matter has been added; this figure is simply a clearer copy of Figure 1 as originally filed and is provided to clarify the presence and absence of the bands. Expression of Bands 1B and 4 is evident in samples obtained from patients age matched with the Alzheimer's disease patients (lanes 5-8, as read from the left) and is not evident in samples obtained from Alzheimer's disease patients (lanes 1-4) or in the sample of pooled NHS (lane 9). Thus, the gel provides evidence that the claimed peptide (SEQ ID NO:1) is differentially expressed between Alzheimer's disease and age matched controls. The gel shown in the figure does not represent new experimentation; the figure shows a clearer image of the original gel made at the time that the experiments described in the instant specification were first carried out.

The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application or any patent issuing thereon.

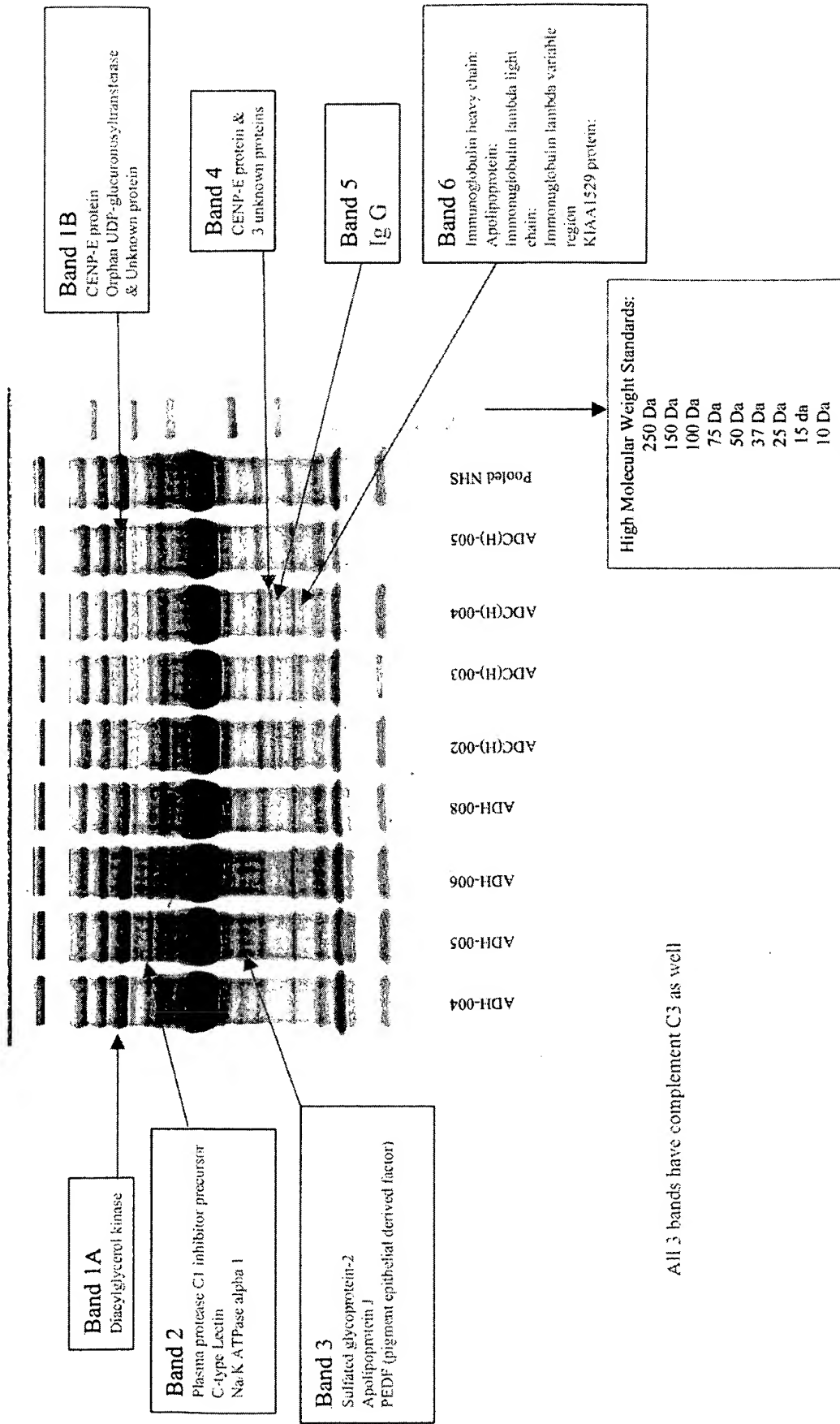
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Ferris H. Lander  
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# DEAE 5 (E) Ad vs. Age Matched AD



All 3 bands have complement C3 as well



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Top Web Results for "linked"

**6 entries found for *linked*.****link**<sup>1</sup>  **Pronunciation Key** (lɪŋk)*n.*

1. One of the rings or loops forming a chain.
2.
  - a. A unit in a connected series of units: *links of sausage; one link in a molecular chain.*
  - b. A unit in a transportation or communications system.
  - c. A connecting element; a tie or bond: *grandparents, our link with the past.*
3.
  - a. An association; a relationship: *The Alumnae Association is my link to the school's present administration.*
  - b. A causal, parallel, or reciprocal relationship; a correlation: *Researchers have detected a link between smoking and heart disease.*
4. A cuff link.
5. *Abbr. li* A unit of length used in surveying, equal to 0.01 chain, 7.92 inches, or about 20.12 centimeters.
6. A rod or lever transmitting motion in a machine.
7. *Computer Science.* A segment of text or a graphical

item that serves as a cross-reference between parts of a hypertext document or between files or hypertext documents. Also called **hotlink**, **hyperlink**.

**v. linked, link·ing, links**

**v. tr.**

1. To connect with or as if with a link: *linked the rings to form a chain*. See Synonyms at join.
2. Computer Science. To make a hypertext link in: *linked her webpage to her employer's homepage*.

**v. intr.**

1. To become connected with or as if with a link: *The molecules linked to form a polymer*.
2. Computer Science. To follow a hypertext link: *With a click of the mouse, I linked to the company's website*.

[Middle English *linke*, of *Scandinavian origin*; akin to Old Norse *hlekk*, \**hlenkr*, from \**hlenkr*.]

**link'ər n.**

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**linked**  **Pronunciation Key** (lɪŋkt)  
**adj.**

1. Connected, especially by or as if by links.
2. Genetics. Exhibiting linkage.
3. Computer Science. Provided with links.

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**linked** (lɪŋkt)*adj.*

Exhibiting linkage.

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**Main Entry: linked**

Pronunciation: 'lɪ [ŋ] (k) t

Function: *adjective*

: marked by linkage and especially genetic linkage &lt;linked genes&gt;

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**linked**

adj : connected by a link, as railway cars or trailer trucks [syn: coupled, joined]

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**linked**

linked: in CancerWEB's On-line Medical Dictionary

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## news and reports

### Mass spectrometry and proteomics

SCOTT D. PATTERSON

Amgen Inc., Thousand Oaks, California 91320-1789

THE 15TH ASILOMAR CONFERENCE on Mass Spectrometry this October was devoted to the role of mass spectrometry (MS) in proteomics. The Asilomar Conference site is in a picturesque national park in Pacific Grove, CA, overlooking the Pacific Ocean. The conference aims to bring together scientists from a cross section of disciplines that are applying MS to an emerging field. This year, that emerging field is proteomics. The term "proteome" was coined by Wilkins et al. (17) in the mid-1990s to describe the protein complement of the genome. The term was first used to describe the 20-yr-old field of two-dimensional gel electrophoresis (2-DE) and quantitative image analysis. 2-DE remains the highest resolution protein separation method available, but the ability to identify the observed proteins was always an extremely difficult problem. MS has been integral to solving that problem. Although improvements in 2-D gel technology had been realized since its introduction, three enabling technological advances have provided the basis for the foundation of the field of proteomics. The first advance was the introduction of large-scale nucleotide sequencing of both expressed sequence tags (ESTs) and, more recently, genomic DNA. The second was the development of mass spectrometers able to ionize and mass-analyze biological molecules and, more recently, the wide-spread introduction of mass spectrometers capable of data-dependent ion selection for fragmentation (MS/MS) (i.e., without the need for user intervention). The third was the development of computer algorithms able to match uninterpreted (or partially interpreted) MS/MS spectra with translations of the nucleotide sequence databases, thereby tying the first two technological advances together. Thus MS played a key role in the passage of 2-DE/image analysis to proteomics.

As a note to readers unfamiliar with MS, the instruments are named for their type of ionization source and mass analyzer (see also Refs. 1, 11, 12). To measure the mass of molecules, the test material must be charged (hence ionized) and desolvated (dry). The two most successful mechanisms for ionization of peptides and proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI the analyte of interest is embedded in a matrix that is dried and then volatilized in a vacuum under ultraviolet laser irradiation. This is a relatively effi-

cient process that ablates only a small portion of the analyte with each laser shot. Typically, the mass analyzer coupled with MALDI is a time-of-flight (TOF) mass analyzer that simply measures the elapsed time from acceleration of the charged (ionized) molecules through a field-free drift region. The other common ionization source is ESI, in which the analyte is sprayed from a fine needle at high voltage toward the inlet of the mass spectrometer (which is under vacuum) at a lower voltage. The spray is typically either from a reversed-phase HPLC (RP-HPLC) column or a nanospray device (19) that is similar to a microinjection needle. During this process, the droplets containing analyte are dried and gain charge (ionize). The ions formed during this process are directed into the mass analyzer, which could be either a triple-quadrupole, an ion trap, a Fourier-transform ion cyclotron resonance (FT-ICR), or a hybrid quadrupole TOF (Qq-TOF) type.

This Asilomar meeting provided one of the largest academic forums in the United States for the presentation and discussion of MS as it is applied to proteomics. As is obvious from the introduction, the initial role MS played was as a protein identification and characterization methodology. However, the role of MS is expanding in this field. Although a series of talks focused on the use of different kinds of MS to identify gel-separated proteins and the various automation technologies applied to perform this in high throughput, several talks also presented alternate approaches. These approaches utilized direct analysis of digested protein mixtures for either identification of the components or quantitative analysis of two different samples mixed together. Specific biological applications were also presented. As described above, a critical component of any MS approach as applied to proteomics is the computational analysis. This report will be divided to focus on these six aspects of MS in proteomics. Where references are known for some of the material presented, they are cited. The program was, however, not entirely limited to MS in proteomics. Prior to the six sections covering the conference core, the first section of this report covers those presentations that were aimed at providing an insight into broader biological and drug discovery processes.

*Proteomics in biology and drug discovery.* The opening lecture, given by Lee Hood (Univ. of Washington), provided an excellent overview of Genomics, Proteomics, and Systems Biology. Hood described the genome project efforts that provide four types of maps: genetic, physical, gene, and sequence. For the human genome,

it is anticipated that 90–95% of all genes will be sequenced sometime next year. This is the first step toward what Hood described as the “Periodic Table of Life.” The different approaches to genomic sequencing and microarray technologies that are able to interrogate the mRNA levels of thousands of genes at a time were described. Hood described proteomics in broad terms as the study of multiplicity of proteins. The information obtained from the various hierarchical levels of biological information (gene, protein, pathways, interconnecting pathways) must be integrated for us to be able to provide a more complete biological picture. For both microarray and proteomics, samples representing the disease process must be obtained. This means that pure cell populations must be microscopically captured from tissues and/or sorted prior to analysis. Therefore, analyses at the mRNA and protein level must be conducted at very low levels and substantial engineering opportunities exist in the biological field to provide the necessary solutions. However, generation of the data is only the first hurdle, as the analysis of the data from a systems perspective then must be undertaken. Hood presented systems biology as the challenge for the 21st century and provided a series of examples of large-scale approaches to biology, from genome sequencing of unicellular organisms, to the sequencing of the T-cell receptor locus, to cancer biology, all of which benefit from such approaches.

Three other presentations were included in the program, to provide a broader background to the utilization of proteomics in drug discovery. Doug Buckley (Exelixis) described the generic view of the drug discovery pipeline, the various “choke” points in the process, and where proteomics could play a role. Of note was the discussion of the changing patent protection landscape, during which Buckley said that full-length cDNA patents were being issued despite the existence of EST patents on portions of these genes. Buckley also predicted that functional data is expected to be required for patents beyond the inferences gained from bioinformatics. The choke points he referred to were target validation, assay development, mechanistic biology, and toxicology. Exelixis is using model organisms (*Caenorhabditis elegans*, *Drosophila*, mouse, and zebrafish) to screen for genes that disrupt/modulate pathways common between man and these organisms. Roles for proteomics included follow-up on targets (direct analysis of protein differences, proteins associated with gene products of interest), assay development [e.g., validation of hits in high-throughput screening (HTS)], and mechanistic biology (e.g., comprehensive analysis of a knockout phenotype). Most importantly, Buckley presented the bottom line that all new technologies must demonstrate their worth by concrete changes in the drug development pipeline (i.e., greater efficiency, better decisions). He predicted that proteomics could provide these benefits at the multiple restriction points referred to above.

Pharmacoproteomics, using 2-DE to profile mechanisms of drug efficacy and toxicity, was presented by Tina Gatlin (Biosource/Large Scale Biology Corpora-

tion). The synergy between mRNA expression profiling (for low-abundance gene products) and protein expression profiling (for posttranslational modifications and subcellular localization) was presented. An exception to this is the search for surrogate markers, where secreted proteins were normally the choice and in which there is no identifiable mRNA source to mirror serum or urine protein expression. The aim of their Molecular Effects Database of 2-DE patterns, obtained from livers of drug-treated rats, is to establish links between expression patterns and toxic endpoints to reveal markers for efficacy and prediction of side effects which can be used for lead selection. In disease models, the hypothesis is that the altered expression pattern could be reversed by treatment with a drug.

The closing presentation of the meeting, given by Jeff Seilhamer (Incyte), presented analyses of the precursor to proteins, mRNA. The staff at Incyte have generated very large EST libraries and from these have estimated the number of genes in the human genome to be 129,769 (based on CpG island estimates, 142,634). They are now sequencing the human genome at a rate of about 1 million reads a month on the Megabace platform with 9 sequencing runs/day. Assembly of the data is being accomplished using Linux on 1,500 CPUs (160 computers) with 75 terabytes of storage capacity. Single-nucleotide polymorphisms (SNPs) are being calculated from their sizable EST collection, and mRNA expression profiling is being achieved using their GEM microarray platform. These data are being integrated with 2-DE proteomics data being generated by their partner Oxford GlycoSciences. This integration of the technologies of genomics and proteomics forms the basis of their drug discovery approach for profiling differences between normal and diseased tissue.

**Computational aspects of proteomics.** Determining the masses of peptides (MS spectra) derived from enzymatic digestion of gel-separated proteins is often the first step in a mass spectrometric-based protein identification strategy. Peptide-mass mapping is the most commonly employed mass spectrometric approach for protein identification from organisms whose genome is completely sequenced (or at least for which the more abundantly expressed genes have been sequenced). The basis of the method is the matching of experimentally determined peptide masses with peptide masses calculated for each entry in a sequence database (using the specificity of the enzyme used to generate the experimental data). How well the experimentally determined masses match with the calculated masses forms the basis of the approach. Ron Beavis (Proteometrics) described how to obtain high-quality data, which even if less, are better than more low-quality data. The use of specific matrices as well as the use of standards with respect to obtaining appropriate data sets for peptide-mass mapping was addressed. Later in the day David Fenyo (Proteometrics) described how to utilize this data in a three-step process as is performed in their WWW-available program, Profound, which uses a Bayesian algorithm (<http://www.proteometrics.com>). The process is as follows:

1) assignment of monoisotopic masses to the raw data, 2) peptide-mass search, and 3) significance testing of the result (4). The last step was presented as the most critical because it is from this that the confidence of the match is derived. This is achieved through calculation of a score frequency function for false positives. This was derived from statistical analysis of the database being searched using random selections of peptide masses from different proteins that are then grouped as synthetic proteins and used in a peptide-mass search of the database in question. This is repeated for a variety of random selections to come up with robust statistics for false positives.

The next level of protein identification is the generation of fragment ion spectra from peptides isolated in the gas phase of the mass spectrometer (MS/MS spectra). Matching of fragment ion spectra follows the same principle as for peptide-mass mapping. Experimentally calculated masses of fragment ions (together with the intact mass of the peptide, and often the specificity of the enzyme used to generate the peptide) are matched with those calculated for isobaric peptides (i.e., same mass as experimentally determined) from entries in sequence databases. Arthur Moseley (Glaxo Wellcome) described how nanoscale capillary LC-MS/MS (where peptides are separated chromatographically before MS/MS) had been automated for identification of gel-separated proteins. The throughput of this 75  $\mu$ m ID capillary system connected to a Qq-TOF mass spectrometer was 20 samples per day at levels to 30 fmol (loaded on gel) for BSA. Moseley continues to develop ultra-HPLC (in some cases combined with variable flow systems) that improve both the speed and resolution of separation. In a Glaxo Organelle Proteomics program, various approaches to protein identification were examined. A comparison of the total number of proteins identified following in situ enzymatic digestion of proteins separated by either high-resolution 2-DE or one-dimensional (usually SDS-PAGE) gel electrophoresis (1-DE) was presented. Only one or a limited number of proteins are present in each of the 2-DE spots, whereas many proteins were present in the 1-DE bands of the enriched Golgi complex. In fact, more proteins were identified from the 1-DE bands than from the 2-DE spots (see below, *Analysis of complex protein mixtures without gel electrophoresis*).

MS/MS spectra derived from tryptic digestions conducted in the presence of equal quantities of  $H_2^{16}O$  and  $H_2^{18}O$ , when combined with subtractive analysis of the two types of spectra, allows de novo sequencing as described by Matthias Wilm (EMBL) (18). By utilizing a Qq-TOF mass spectrometer, peptides containing both COOH-terminally incorporated stable isotopes and just the isoform containing the  $^{18}O$  could be selected for fragmentation from the mixture. Subtraction of the  $^{18}O$  spectrum from the  $^{16}O$ : $^{18}O$  spectrum reveals only  $^{16}O$   $\gamma$ -series ions. Often, a complete ion series is obtained. The method has proved feasible in their hands when 1 pmol of protein is present in the gel (1/4 of this amount can be successfully analyzed with standard digest conditions).

*Automated identification of gel-separated proteins by mass spectrometry.* Following quantitative analysis of 2-DE patterns, the next step is the identification of all protein spots that display differential expression. Andrew Gooley (Proteome Systems) described the approaches they are employing for quantitative analysis using 2-DE. This included the following: sample preparation (sequential detergent extraction with aminosulfobetaine-14), narrow-range immobilized pH gradient (IPG) with mini-gels for the 2nd dimension, through to the robotic system that they have codeveloped for spot excision, liquid handling (peptide extraction and reverse-phase bead cleanup and storage) and peptide-mass fingerprinting by MALDI-MS. Apart from the throughput of the robotic system, diminished contamination from keratin and more reproducible spotting of samples for MALDI-MS is a highly desirable feature of automation. Hans-Werner Lahm (Hoffmann-La Roche) described the high-throughput system they use for automated spot excision from 2-DE, digestion (with low-salt buffer to eliminate the need for cleanup), and spotting for automated MALDI-MS. Lahm also described the computational aspects of operating such a system in high-throughput mode for long periods of time, including automated database search routines for users distributed throughout the world at other Roche sites. They are investigating the use of stable isotope labeling ( $^{14}N/^{15}N$ ) followed by mixing of each sample prior to 2-DE for direct quantitation of relative expression differences from the MALDI-MS spectra of individual protein spots. The system averages 1,000 spots to spectra per day (including downtime).

David Arnott (Genentech) described automation of in-gel digestions following analysis of differentially regulated proteins from 2-DE. Arnott described the trapping cartridge approach that was required to analyze extracted peptides from the DigestPro robot (currently 30 sample spots, but upgradeable to 96) by microcapillary LC-MS/MS. They aimed to automate as much of the sample processing as possible with automated liquid handling from the digestion robot to the data-dependent LC-MS/MS (capable of handling 40 samples per day) using an ion-trap mass spectrometer followed by auto-database searching using Sequest (3). The system is capable of analysis of subpicomolar quantities of protein from silver-stained gels.

*Advances in separations and mass spectrometers.* Accurate mass analysis of intact proteins using an 11.5-T FT-ICR coupled with a capillary electrophoresis (CE) instrument was demonstrated by Richard Smith (Pacific Northwest National Laboratory) as a means of proteome analysis. Through the use of stable isotope labeling of one sample and running that sample with an unlabeled sample provides the possibility to measure protein expression ratios. To identify the proteins that display different ratios, dissociation in the FT-ICR-MS to yield mass tags is possible. Having intact mass information as well as identification allows post-translational modifications to begin to be investigated. The mass accuracy obtainable by this FT-ICR-MS was said to be <0.75 ppm which allows the generation of

accurate mass tags for tryptic peptides. In many cases this may be sufficient for protein identification (at least for an organism like *C. elegans*). In some cases, MS/MS may be required, but once performed it would not have to be repeated. Another possibility is the identification of cysteine-containing peptides at a mass accuracy of 1 ppm, which was said to be sufficient for identification. Another possibility being explored with this instrument is multiplexed MS/MS, where up to 7 ions could be isolated at once and the MS/MS spectrum could be deconvoluted for each selected ion (requires accuracy of <10 ppm). This will be tried with online separations in the near future.

Marvin Vestal (PE BioSystems) described his continuing efforts in MALDI-MS instrument design. The attributes he is aiming for include sensitivity, specificity (resolution, mass accuracy, selective ionization), speed, accuracy, dynamic range, and mass range. The sensitivity will always be limited by chemical noise, but the aim is to reduce the limitations of ionization and data handling. Vestal would like to achieve a sensitivity of 1 fmol with a data acquisition rate of 1 spectrum/s. The instrument he is designing to achieve these aims is a MALDI-TOF/TOF-MS. This system has an ion gate (with 500 resolution and no loss of sensitivity) after the collision cell so that metastable ions created after reacceleration are removed. Although this system is in the early stages of development, the data shown demonstrate that this instrument is meeting most of the stated objectives.

The hybrid quadrupole TOF (Qq-TOF) mass spectrometer developed a few years ago (9), which has now been commercialized, utilizes an ESI for ionization (10). Both Ken Standing (Univ. of Manitoba) and Brian Chait (Rockefeller Univ.) described the use of a MALDI ion source for introduction of ions into a modified commercial Qq-TOF, thus taking advantage of both the high-efficiency ion production of the MALDI and the ion isolation/fragmentation of the quadrupole system with a TOF mass analyzer. Standing presented data showing sensitivity of purified standards (e.g., Substance P) in the 70 amol range (1-min acquisition) for MS and 7 fmol for MS/MS with 10,000 resolution. This instrument offers similar advantages to the TOF/TOF described above.

Online MS analysis of capillary electrophoretic or chromatographic separations of peptide (or proteins) is most often achieved using ESI-MS. Barry Karger (Barnett Institute) described how very small quantities of peptides/proteins could be separated and analyzed using vacuum deposition onto Mylar audio tape for subsequent coupled MALDI-MS analysis. The approach had so far been multiplexed with the effluent of 12 capillaries being deposited under vacuum onto the tape. The approach is designed for high-throughput separations and mass analysis.

Proteomic analyses often employ 2-DE, but David Lubman (Univ. of Michigan) described a liquid-phase 2-D separation of proteins utilizing a novel MS. The requirements of his mass spectrometer were high sensitivity, low duty cycle, and fast response. He designed and built an ion trap to capture ions from the CE

coupled to TOF-MS. The 2-D liquid-phase separation consisted of nonporous silica bead RP-HPLC (which provided good resolution <50 kDa) that was conducted at high pH followed by CE and MS. Whole cell lysates were analyzed with this system, and some of the data obtained were presented.

**Biological applications.** Brian Chait (Rockefeller Univ.) presented the culmination of an enormous amount of work at both the protein chemistry (mass spectrometry) and cell biology levels. The nuclear pore complex (NPC) in yeast is a massive structure (1,000 Å across with 8-fold symmetry) that regulates protein transport in/out of the nucleus. The first step in understanding this structure was to purify the complex and then identify every protein present. The protein fraction was separated by several different chromatographic steps followed by SDS-PAGE from which every visible band was excised and analyzed by MALDI-IT-MS. This was an especially daunting task as the NPC when isolated contains a snapshot of the proteins transiting the NPC at that point in time. Hence, of the 174 proteins identified, 29 were nucleoporins and only 14 were shown to be present in the NPC. These 14 proteins were characterized as being present in the NPC by a variety of techniques. Protein A (4.5 repeats of the Fc binding region) fusions with the proteins of interest were generated, and immunohistochemistry was performed on cells transfected with these constructs. Electron microscopy of hundreds of NPCs following transfection allowed stoichiometry and symmetry (nuclear/cytoplasmic/asymmetric) to be determined. Subcellular fractionation and high-pH extractions were also performed to further characterize localization biochemically. This elegant study has allowed a testable model for nuclear transport to be constructed.

Two examples of the utility of analysis of unfractionated or partially fractionated complex protein mixture digests (see next section) were presented by Scott Patterson (Amgen Inc.). As a first step in the understanding of the interchromatin granule clusters (IGC), a nuclear organelle which is a major site of mRNA splicing. Samples enriched in this structure were digested with trypsin, and the complex mixture of peptides was analyzed by data-dependent LC-MS/MS (8). Some proteins known to be present in these structures were identified together with 19 novel genes (including ESTs). Three of the genes were confirmed to be present in the IGC by immunohistochemistry of cells transfected with yellow fluorescent protein (YFP)-fusion constructs with counter staining of splicing factors. The other study presented identified 108 proteins present in a protein fraction obtained from isolated mitochondria treated with atractyloside [mimicking in vitro the permeability transition pore complex (PTPC) which occurs during apoptosis] (13).

Analysis of immunoprecipitates using a new affinity strategy was presented by Gitte Neubauer (EMBL). The new strategy is referred to as tandem affinity purification (TAP) and was developed by colleagues at EMBL (15). The system utilizes a double tag for higher



specificity and much reduced background. The human spliceosome immunoprecipitated under normal conditions (see Ref. 5 for same approach with yeast trsnRNP) and using the TAP method were compared, demonstrating the utility of this approach.

The common theme of all of these applications is that MS was utilized early on to provide rapid and accurate protein identifications. The genes identified could then be further analyzed to attempt to determine their function.

The use of MS to identify proteins from 2-DE gels was also described by Al Burlingame (Univ. of California, San Francisco) and Reid Townsend (Oxford GlycoSciences). Burlingame described their work to identify protein targets of acetaminophen during acute toxicity and the intricacies of such analyses (14). Townsend described an Oxford GlycoSciences and Pfizer collaboration to separate by 2-DE and identify proteins from cerebrospinal fluid (CSF) in a study aimed at identifying markers for Alzheimer's disease. CSF is a compartment isolated by the blood-brain barrier but it is not just a filtrate of blood. It is produced by the choroid plexus and has a total central nervous system volume of about 90–150 ml that is turned over a few times per day. Comparative analysis of matched plasma CSF samples (in addition the normal/diseased samples) revealed that key plasma proteins (e.g., albumin, transferrin, IgG) showed markedly different relative ratios between plasma and CSF. For effective 2-DE analysis of these samples, a selective removal of albumin, IgG, transferrin, and haptoglobin had to be developed. This was accomplished by affinity depletion. Interestingly, many features in a 2-DE separation are albumin fragments (in fact, 4% of total features). Their study included 512 samples from 228 patients and resulted in 1,131 features (spots) being annotated. Potential markers of Alzheimer's disease were said to be identified.

Separate from the MS identification issues covered in most of the meeting, Kerstin Strupat (Univ. of Muenster) presented her work on MS analysis of noncovalent complexes. Here the challenge is to transfer noncovalent interactions that occur in the condensed phase to the gas phase. ESI-MS has been shown by a number of groups to work, but MALDI-MS analysis has proved more difficult. Examples of MALDI-MS analysis of noncovalent protein:protein (streptavidin tetramer and the macrophage migration inhibitory factor related proteins MRP-8 and MRP-14) and protein:ligand (aldose reductase:NADP) interactions were presented. Interestingly, analysis of the first laser pulse during a MALDI-MS analysis often allows investigation of noncovalent interactions that are not observed during subsequent pulses (16).

*Analysis of complex protein mixtures without gel electrophoresis.* The first stage of many proteome projects is the identification of the components comprising the system under study. This is of course the first step in understanding any biological system. As described above, an increasing (but still limited) number of laboratories have access to robotic systems requisite for the analysis of large numbers of spots from 2-DE.

However, a trend in the field is emerging toward the elimination of the high-resolution protein separation step prior to protein identification by MS. In this approach, the entire enriched protein fraction is enzymatically digested (usually with trypsin), and the resulting complex peptide mixture is subjected to data-dependent LC-MS/MS. In this approach the peptides are separated by both hydrophobicity (RP-HPLC) and charge ( $m/z$  in the mass spectrometer) prior to ion selection by the MS control software (hence, data dependent). At this meeting, presentations from five groups demonstrated the utility of the approach to identify components of complex mixtures.

Analysis of immunoprecipitated proteins or enriched protein fractions (e.g., Golgi complex) by either gel electrophoresis followed by in-gel digestion and MS or digestion of the entire protein fraction and analysis by data-dependent LC-MS/MS using a Qq-TOF was described by Jyoyti Choudhary (Glaxo Wellcome). Batched MS/MS spectra were searched using the Mascot program (<http://www.matrixscience.com>). Data presented showed that if the immunoprecipitate was clean, then direct digestion of the mixture proved slightly more successful than analysis of gel-separated proteins. When an enriched Golgi complex from rat liver was separated by either 2-DE (135 spots) or 1-DE (77 bands) and in-gel digested followed by LC-MS/MS, significantly more proteins were identified from the 1-DE separation.

David Arnott (Genentech) described the proteomics component of Genentech's Secreted Protein Discovery Initiative, which also includes genomic, signal trap, expression, and functional analysis. Arnott evaluated three methods to identify proteins secreted from human umbilical microvascular endothelial cells (HUMECs) into 60 ml of serum-free media; 2-DE and 1-DE (with/without staining) followed by in-gel digestion, and direct digestion of the entire protein mixture. Digests were analyzed using the microcapillary system described above. Interestingly, direct digestion followed by data-dependent LC-MS/MS identified the most proteins, but all three methods were complementary in their hands (21 proteins identified by all three methods but no completely novel gene products).

Analysis of serum fractionated using the Cohn pH/ethanol precipitation protocol followed by digestion of the entire fraction prior to data-dependent LC-MS/MS was described by Karl Clauser (Millennium Pharmaceuticals) in the context of the studies of differences between wild type and ApoE  $-/-$  mice. Clauser also presented the bioinformatics flow for data handling, which utilizes a variant of the publicly available MS-Tag (<http://prospector.ucsf.edu/>) for protein identification and a de novo sequence interpretation program referred to as SHERENGA (2). Their stated aim is for searching to keep up with the LC-MS/MS. They have also been experimenting with the IEX ion-exchange protocol developed by Andy Link (7) as a means of decreasing the complexity of the sample and reducing the number of singly charged and highly charged ions as these are less likely to be identified. In one IEX

fraction, 87 plasma proteins were identified in a single run compared with 66 from an unfractionated sample.

Scott Patterson (Amgen) described Amgen's proteomics efforts, now in the third year. They are employing data-dependent LC-MS/MS of complex protein mixture digests. The stated aim is to reduce the complexity such that in an ideal situation only one peptide for each protein in the mixture is fragmented during LC-MS/MS. To achieve this aim, various affinity methods can be employed, and the use of cysteinyl peptide capture using either thiopropyl Sepharose or a biotin alkylating reagent, *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (HPDP-biotin), was described (13). The former was used in a large-scale analysis of urinary proteins where digestions of the unfractionated starting material, albumin/IgG depleted, or cysteinyl peptide captured or noncaptured were analyzed. The samples were analyzed with replicate LC-MS/MS runs using narrow mass ranges for ion selection for each run, thereby increasing the number of unique spectra selected for fragmentation. This analysis resulted in the identification of over 200 proteins, including a number of uncharacterized nucleotide sequences (e.g., ESTs). Smaller scale analyses are described above, in one case [soluble intermembrane proteins (SIMP)] utilizing cysteinyl peptide capture to identify more proteins than with no fractionation. Data handling for this high-throughput effort was also briefly described. A number of the fractions being analyzed have some of the same components; therefore, to enhance the identification process, spectral matching of the database (>5 million spectra) is performed. This links identical spectra and therefore reduces the redundancy associated with re-searching already identified spectra.

*Quantitative analysis of two samples without electrophoresis.* MALDI-MS, using the surface enhanced laser desorption ionization (SELDI) surface, to search for disease markers in biological fluids was presented by Scot Weinberger (Ciphergen Biosystems). In this approach, defined chemical/biochemical surfaces are utilized to allow fractionation of proteins from biological fluids in a reproducible manner. This reproducibility allows comparisons between different samples to be made. Weinberger described the search for markers of benign prostatic hyperplasia that, like prostate cancer, displays elevated prostate specific antigen (PSA) levels. The fraction exhibiting a difference between these samples was able to be enzymatically digested, and a number of peptides were generated. These were able to be fragmented using the MALDI Qq-TOF of Standing, described above. It appears as though there is a difference in the relative level of a seminogelin fragment between these two diseases, providing a potential differential marker. The method is sensitive but apparently limited to analysis of proteins less than about 20 kDa (a range not well characterized by 2-DE).

A combination gel/MS approach referred to as a "virtual 2-D gel" was presented by Phil Andrews (Univ. of Michigan). In this approach, proteins are separated by charge using thin-layer isoelectric focusing (IEF), and this gel is then subjected to MALDI-MS. By

rastering through the entire IEF gel, a composite display of all acquired MALDI-MS spectra can be generated (hence, the virtual 2-DE). Such analyses would provide very accurate mass measurements, greatly assisting in posttranslational modification analyses as well as potentially quantitation.

Karl Clauser (Millennium Pharmaceuticals) described their efforts at utilizing already existing LC-MS/MS data to attempt to gain some quantitative/qualitative information as to differences between samples. Differences in serum protein levels between wild-type and ApoE  $-/-$  mice have been examined using this approach, which compares the MS ion current from peptides identified between LC-MS/MS runs of each sample. Comparison between runs is a difficult task, but data suggested that there is sufficient confidence to state a significant difference if there is a difference of a factor of 3 between some components of the samples.

An LC-MS/MS-based system was described by Steve Gygi (Univ. of Washington) for quantitative analysis of complex mixtures. The technology is referred to as isotope-coded affinity tag (ICAT) (6). The ICAT reagent described here is composed of three units: an affinity reagent (biotin), a linker region (one of two forms), and a reactive group (a thiol-specific reagent, iodoacetic acid). The linker region encodes the mass difference, with the light version having 8 hydrogens and the heavy version having 8 deuteriums. Thus the mass difference is 8 mass units (doubly charged ions will have an  $m/z$  difference of 4). Following reduction and alkylation of each of the two protein samples with one of the two reagents, the two samples can then be mixed together. All subsequent manipulations are performed as a mixture, culminating in tryptic digestion of the complex sample and capture of the cysteinyl peptides on avidin. The bound peptides are released and analyzed by LC-MS/MS, revealing paired signals of peptides. Calculation of areas under the peak for each paired ion from the LC-MS data provides an accurate record of the relative quantities of the proteins from each starting sample. The MS/MS spectra allow identification of the peptides. The approach was elegantly demonstrated with yeast grown on either galactose-containing media or ethanol-containing media. Proteins expected to be differentially regulated were observed, and, highlighting the advantages of analysis at the protein level as opposed to the mRNA level (e.g., microarray), alcohol dehydrogenase-1 (ADH1) was found to be oppositely regulated (as expected) to ADH2, to which it is 95% homologous. This is a very promising approach for quantitative analysis of complex protein mixtures.

A number of interesting posters were also presented at the meeting, and some of the presenters were given the opportunity to "advertise" their posters. These dealt with the same range of subjects presented in the oral sessions.

*Conclusion.* The organizers Ruedi Aebersold and John Stults brought together an excellent program for this meeting, with essentially all major laboratories in

this field being represented. The field has grown enormously over the past few years, and advancements presented at this meeting indicate an optimistic view of the future for proteomics. This very successful meeting provided the 162 attendees with the state-of-the-art in mass spectrometry and proteomics.

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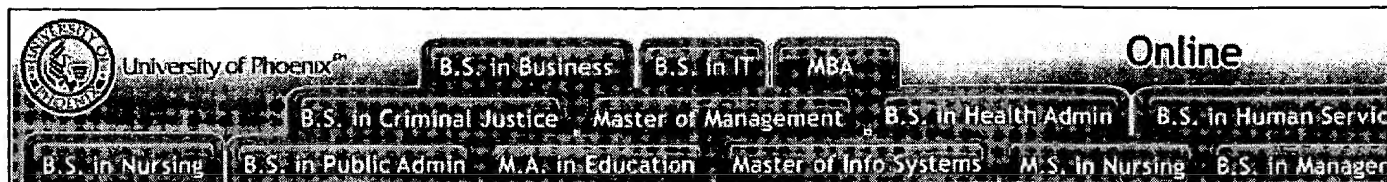
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  - b. A tombstone.
  - c. A milestone.
2. An implement, especially a felt-tipped pen, used for marking or writing.
3. One who marks objects, especially for industrial purposes.
4. One who grades student papers.
5. Sports.
  - a. A device, such as a line, stake, or flag, set on a playing field and showing the playing or scoring position.
  - b. A player who guards an opponent, as in soccer.
  - c. An official in certain court games, such as squash, who mainly judges whether the ball is out of play and whether a fault or let has occurred.
6. *Games*.

- a. One that keeps score in various games.
- b. A score in a game.
7. *Slang*. A written, signed promissory note.
8. A genetic marker.
9. *Medicine*. A physiological substance, such as human chorionic gonadotropin or alpha-fetoprotein, that when present in abnormal amounts in the serum may indicate the presence of disease, as that caused by a malignancy. Also called **biomarker**.
10. *Linguistics*. An element that indicates grammatical class or function; a derivational or inflectional morpheme.

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*n.*

1. One that marks or serves as a mark.
2. A physiological substance, such as human chorionic gonadotropin or alpha-fetoprotein, that may indicate disease when present in abnormal amounts in the serum, as that caused by a malignancy. Also called *biomarker*.
3. A genetic marker.

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**2** : GENETIC MARKER called also *marker gene*

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**marker**

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PubMed Central**Elevated 5-S-cysteinyl dopamine/homovanillic acid ratio and reduced homovanillic acid in cerebrospinal fluid: possible markers for and potential insights into the pathoetiology of Parkinson's disease.****Cheng FC, Kuo JS, Chia LG, Dryhurst G.**

Department of Medical Research and Geriatrics Medical Center, Taichung, Taiwan, Republic of China.

High-performance liquid chromatography with electrochemical detection has been employed to analyze ultrafiltrates of cerebrospinal fluid of Parkinson's Disease (PD) patients and age-matched controls for the dopamine (DA) metabolites homovanillic acid (HVA) and 5-S-cysteinyl dopamine (5-S-CyS-DA). The mean level of HVA in the CSF of PD patients, measured 5 days after withdrawal from L-DOPA therapy, was significantly lower than that measured in controls. By contrast, mean levels of 5-S-CyS-DA were not significantly different in the CSF of PD patients taking L-DOPA (PD-LT patients) the same patients 5 days after discontinuing this drug (PD-LW patients) or controls. However, the mean 5-S-CyS-DA/HVA concentration ratio was significantly ( $p < 0.05$ ) higher in the CSF of PD-LW patients compared to controls. Although the PD patient population employed in this study had been diagnosed with the disease several years previously and had been treated with L-DOPA for prolonged periods of time the results of this study suggest that low CSF levels of HVA and a high 5-S-CyS-DA/HVA ratio together might represent useful markers for early diagnosis of PD. The high 5-S-CyS-DA/HVA ratio observed in the CSF of PD-LW patients also provides support for the hypothesis that the translocation of glutathione or L-cysteine into neuromelanin-pigmented dopaminergic cell bodies in the substantia nigra might represent an early event in the pathogenesis of PD.

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## Chromogranin A in cerebrospinal fluid: a biochemical marker for synaptic degeneration in Alzheimer's disease?

**Blennow K, Davidsson P, Wallin A, Ekman R.**

Department of Psychiatry and Neurochemistry, University of Goteborg, Molndal Hospital, Sweden.

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Biochemical markers for AD would be of great value both to improve the clinical diagnostic accuracy in scientific studies and to increase the knowledge of the pathogenesis of the disorder. One of the main features of AD is a degeneration of synapses. Therefore, we examined if chromogranin A (CrA), the major protein of large dense-core synaptic vesicles, in cerebrospinal fluid (CSF) may be of value as a biochemical marker for the synaptic function in AD. The mean concentration of CrA in CSF was about 7.5 times higher than its concentration in serum, and there was no significant correlation between CSF-CrA and the blood-brain barrier function (measured as the CSF/serum albumin ratio), nor between CSF-CrA and serum-CrA. These findings suggest that the major portion of CSF-CrA is locally produced within the CNS. There were no significant differences in CSF-CrA between the AD ( $n = 29$ ), vascular dementia ( $n = 13$ ), and age-matched control ( $n = 9$ ) groups ( $99.9 \pm 58.9$  ng/ml,  $108.0 \pm 69.4$  ng/ml, and  $115.1 \pm 44.4$  ng/ml, respectively). However, when the AD group was subdivided into AD type I ( $n = 12$ ) and AD type II ( $n = 17$ ), a lower concentration of CSF-CrA was found in AD type I ( $72.8 \pm 28.9$  ng/ml) compared with controls ( $115.1 \pm 44.4$  ng/ml),  $p < 0.02$ , and compared with AD type II ( $119.1 \pm 67.5$  ng/ml),  $p < 0.05$ , while CSF-CrA did not significantly differ between AD type II and controls. These findings suggest that CSF-CrA has a potential as a biochemical marker for the synaptic degeneration in AD type I, and gives further support for the relevance of identifying the AD type I (pure AD) subgroup in scientific studies.


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Here we show that suppression of synthesis of the microtubule motor CENP-E (centromere-associated protein E), a component of the kinetochore corona fibres of mammalian centromeres, yields chromosomes that are chronically mono-orientated, with spindles that are flattened along the plane of the substrate. Despite apparently normal microtubule numbers and the continued presence at kinetochores of other microtubule motors, spindle poles fragment in the absence of CENP-E, which implicates this protein in delivery of components from kinetochores to poles. CENP-E represents a link between attachment of spindle microtubules and the mitotic checkpoint signalling cascade, as depletion of this motor leads to profound checkpoint activation, whereas immunoprecipitation reveals a nearly stoichiometric association of CENP-E with the checkpoint kinase BubR1 during mitosis.

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## The cytoskeleton in Alzheimer disease.

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The strongest physical correlate with the severity of dementia in Alzheimer's disease and its most rational cause are the loss of neocortical and hippocampal synapses. Evidence, showing that beta-amyloid causes that loss is weak despite the popularity of that hypothesis. Other changes can better explain that damaging phenomenon. Axonal terminals are dependent on axoplasmic flow, and that function requires intact microtubules and the motor proteins kinesin, dynein and dynamin. It has been known since the earliest electron microscopic studies of AD that neuronal microtubules are lessened in number. Tubules are normally in equilibrium with unpolymerized tubulin, and the stability of the formed elements is dependent on normal binding of tau to the tubule. But, as is well known, tau is abnormally hyperphosphorylated in AD leading to tangle formation and to dissolution of the tubules. Tangles are insufficient in number to account for the cortical loss of neurons and synapses, but hyperphosphorylated tau in the unpolymerized pre-tangle state undoubtedly plays a role. Abnormalities in the motor proteins are now being investigated (some have already been found) and these too would contribute to the loss of synapses in AD by way diminished axoplasmic flow.

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